RESPIRATORY SYNCYTIAL VIRUS WITH A GENOMIC DEFICIENCY COMPLEMENTED IN TRANS

Field of the invention

The present invention relates to the field of vaccination, and more specifically to vaccines against disease caused by pneumoviruses such as e.g. Respiratory Syncytial Virus (RSV). The invention pertains RSV virions carrying an RSV genome in which a gene that is essential for infectivity has been inactivated, while the corresponding wild type gene-product is complemented *in trans* to the virion. The invention further relates to methods for the production of such RSV virions and to their use in vaccines and methods for vaccination against pneumoviruses.

Background of the invention

Human respiratory Syncytial virus is classified in the genus Pneumovirus, family Paramyxoviruses. It is a major cause of severe lower respiratory tract disease in infants, the elderly and in immunocompromised individuals. It is also an important factor in upper respiratory tract disease in older children and adults. Currently there is no effective h-RSV vaccine available in the art.

RSV is an enveloped RNA virus that expresses two major antigens at its surface: the attachment protein G and the fusion protein F. Both proteins appear to invoke protective antibodies. G is the determinant of the two known h-RSV subgroups A and B. Antigenic differences can be found within the two groups. The G protein shows a high degree of variation with only 53 % amino acid homology between groups A and B and up to 20% differences in G protein sequences within group A (Mufson 1988, Cane 1991).

Passive immunisation with RSV-enriched immunoglobulin (Respigam) or synthetic humanised monoclonal antibodies against F (Palivizumab) is currently used to treat and protect neonates of certain predispositions (e.g. premature birth) against RSV infection (Robinson 2000, Greenough 2000). RSV pathology has two major aspects: cell damage caused by the virus itself and tissue damage caused by the overreacting immune system. The latter is a highly complicating factor in vaccine design.

RSV infections are seasonal, limited to the winter period and peak in the Northern Hemisphere around the end of the year. RSV infects every child before the age of two, in many cases twice. Older individuals on average are infected every other year, depending on the setting; people in close contact with infants and young children have a 50% risk. The virus spreads by close contact, in droplets or through contaminated surfaces. RSV is not efficiently spread through aerosols; the virus particles are relatively unstable. Internal spread of the virus from the upper respiratory tract (URT) to the lower respiratory tract (LRT) occurs predominantly by inhalation of virus particles produced in the URT epithelium during primary infection. Spread through syncytium formation (one of the pathological properties of the virus, which gave it its name) can not be ruled out and may play a secondary role in LRT infection.

In general, RSV pathology starts in the URT; the port of entry is the nose and to a lesser extent the eyes – not the mouth. When restricted to URT tissues, disease is limited to common cold, although in adults sometimes severe. However, when the virus can reach the LRT, bronchiolitis and pneumonia can ensue in unprotected individuals. In young infants, this can be life threatening, approx. 1/100 will require hospitalisation and mechanical ventilation, out of these 1% may die. In the elderly, RSV-induced LRT disease is a major cause of hospitalisation; it is suspected that RSV causes 25% of flu-like diseases.

The immune response to RSV is complex. In general, exposure to h-RSV will build up a response that protects against LRT disease. This response wanes with older age, causing the higher susceptibility to RSV of the older population. Effective long lasting protection against URT disease appears not feasible: re-infection is very common, even within the same season and this is not caused by viral variation. Protection against RSV infection involves antibodies against viral proteins F and G circulating in the blood, which can prevent LRT disease. URT infection can be controlled by mucosal antibodies against F and G, but these have a limited life span. CD8+ T cells against as yet unidentified viral proteins are required to clear the virus from infected tissues, but they appear to be short-lived or inefficiently recruited from their reservoirs. Most likely, this is caused by RSV-expressed factors, possibly encoded in the G gene (Srikiatkhachorn, 1997a).

An important aspect of RSV disease is immune enhancement of pathology. In limited cases the cellular immune response can exacerbate RSV disease by the action of

cytokines on infected tissues released from excessively attracted granulocytes. Host predisposition is involved in this reaction, but possibly also the timing of first RSV infection after birth. Unfortunately, early vaccine trials with formalin-inactivated RSV showed that in these vaccination settings immune enhanced pathology upon wt infection was prevalent (Kim 1969). Factors contained in RSV appear to be responsible for this phenomenon and were apparently released by formalin treatment. In the 40 years since then, it was gradually shown that the viral G protein is the predominant mediator of these problems, but the mechanism remains unclear (Srikiatkhachorn 1997b). In any case, vaccination with a G protein out of the context of the virion (i.e. in inactivated virus preparations, as expression product not properly embedded in a membrane or in the form of peptides) seems to be causing immune enhancement in model systems. Thus, although G contributes to some extent to RSV immunity, its properties also complicate vaccine design.

Initial live RSV vaccine candidates included cold passaged or temperature-sensitive mutants. The former have been attenuated by culturing at decreasing temperature, leading to dependency on low temperatures for growth, whereas the latter mutants have been made dependent on a specific, usually higher temperature for replication by chemical or radiation mutagenesis. These live virus vaccine candidates appeared to be either under- or overattenuated (Crowe 1998).

Subunit vaccine candidates are derived from either the RSV-F or the G protein, being the main targets for neutralising antibodies. A candidate subunit vaccine, PFP2, purified F protein, is safe in RSV-seropositive patients, but it did not provide full protection against LRT infection and associated disease (Gonzalez 2000). Another subunit vaccine approach is BBG2Na, which consists of a polypeptide, comprising amino acid 130-230 of h-RSV-G, fused to the albumin-binding domain of streptococcal G protein (Power 1997). BBG2Na induces a T helper type 2 response in neonatal mice, and does not elicit lung immunopathology (Siegrist 1999). There is no data yet on protection. The use of new adjuvants for a balanced humoral and cellular immune response are currently under investigation in animal models (Plotnicky 2003).

The use of plasmid-DNA vectors encoding RSV-F and G antigens as vaccine candidates has been studied in animal models. These vaccines induce protective responses in rodents (Li 2000), but in one study RSV-F DNA vaccine candidate immunised mice developed a slightly enhanced pulmonary inflammatory response

following challenge with wt virus (Bembridge 2000). The feasibility of the use of plasmid DNA vaccines in humans is not yet known and it will likely take at least 15 years before this approach is sufficiently studied and - more importantly - accepted, particularly for neonates. Candidate vaccines based on vector delivery systems are constructed of live recombinant vectors expressing RSV proteins. For example, recombinant vaccinia virus expressing RSV-F and G provided protection in mice, but lacked this effect in chimpanzees (Collins 1990). The question is whether these systems are safe (notably vaccinia virus) and feasible in the light of existing (maternal) antibodies against poxviruses in the community and the main target group being neonates.

Several vaccine candidates are based on recombinant live RSV, generated by reverse genetics. One line of study focuses on attenuating these viruses by introducing the individual or combined mutations responsible for cold-adaptation and temperature-sensitivity into the recombinant virus. None of these vaccine candidates were usable, because of either over- or underattenuation. Another line of study focuses on deletion of one or more viral non-structural genes. Limited data are available on the behaviour of these viruses in model systems (Jin 2003).

An alternative approach to RSV vaccine development is the use of bovine RSV. A chimeric bovine RSV with either the human F protein alone or both the human F and G protein was evaluated for its efficacy in chimpanzees. This vaccine candidate was restricted in replication to such a degree that animals were not protected after wild type h-RSV challenge (Buchholtz 2000).

Thus, currently there is no effective h-RSV vaccine available in the art. All RSV vaccine candidates that have been tested in animal models are unusable in humans. There is thus a long felt need in the art for RSV vaccines that are both effective and safe and it is an object of the present invention to provide for such vaccines.

Description of the invention

Definitions

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one

of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The term "virion" as used herein refers to a virus particle that contains the nucleocapsid protein, the viral genome and the replicase complex in a lipid envelop that contains the viral structural glycoproteins.

The terms "infectivity of a virus", "infectious virus", "infectious virus particle" or "infectious virion" denote viruses, virus particles or virions that are capable of entering suitable host cells and initiating a virus replication cycle, whether or not this leads to the production new virus that is infectious.

Detailed description of the invention

In a first aspect the present invention relates to a virion of a pneumovirus. The virion comprises a viral genome that has a mutation in a gene coding for a protein that is essential for infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein in a form and in an amount that is required for infectivity of the virion.

The pneumovirus preferably is a Respiratory Syncytial Virus (RSV), more preferably a human or bovine RSV. The human RSV may either be a subgroup A or B virus, and preferably is a clinical isolate, more preferably an isolate that has not been extensively passaged *in vitro*(preferably passaged less than 10, 8, 6 or 5 times as descrined in the Examples). Therefore, any RSV strain or isolate may be used in the context of the present invention, whereby is understood that the invention is only exemplified by means of the particular human RSV isolate 98-25147-X, referred to as RSV isolate X. Further preferred is that virus is a recent clinical isolate whereby recent is defined as being first isolated less than 10, 8, 6, 4, 3, or 2 years ago. It will be understood that although the nucleotide sequences in the virion do not need to correspond to those of the recent isolate, preferably, the amino acid sequences of the proteins present in the virion of the invention are identical to the proteins as they occur in a recent clinical isolate.

The viral genome comprises at least one mutation in at least one viral gene coding for a protein that is essential for infectivity of the pneumovirus, whereby the infectivity of the virus is as defined above. Thus, the protein that is essential for

infectivity of the pneumovirus is a protein that is essential for the capability of the virion of the invention to enter a suitable host cell and initiate a viral replication cycle, whereby the replication cycle does not necessarily lead to the production of new infectious virions. In preferred virions of the invention the mutation causes the virion to lack infectivity *in vivo*, i.e. in a suitable host organism, whereby the virions may still be infectious for suitable host cells cultured *in vitro*.

In a preferred virion of the invention, the mutated gene that codes for a protein essential for infectivity of the pneumovirus, is a gene, which codes for a structural protein of the virus. A structural protein of a pneumovirus is herein understood to be a protein that is present in virions of wild-type infectious virus. Preferred genes coding for structural proteins to be mutated in the virions of the invention are the genes coding for the attachment protein G and/or the fusion protein F, whereby the G protein is most preferred. Deletion and/or functional inactivation the gene coding for G protein serves several purposes and prevents a number of problems and complications of current RSV vaccine candidates. One purpose is vaccine safety: RSV without G protein is highly attenuated in its host (Karron 1997, Schmidt 2002) because it will not be able to efficiently infect host cells. One complication is that the G protein is strongly implicated in causing undesired immunological responses, including enhanced immune pathology (Alwan 1993, Srikiatkhachorn 1997b) and possible skewing of the immune system towards an allergy (and asthma-) prone state under certain genetic predispositions (Openshaw 2003, Peebles 2003). This will be prevented by deletion or inactivation of the G gene. A pneumoviral virion of the invention comprising a viral genome that has an inactivating mutation in the gene coding for a G attachment protein, and comprising the G attachment protein in a form and in an amount that is required for infectivity of the virion is referred to as a "ΔG+G" (pneumo)virus or virion. Similarly, the virion that has the inactivating mutation in the gene coding for a G attachment protein, but which is not complemented *in trans* with a functional amount of G protein is referred to as a "ΔG" (pneumo)virus or virion.

The pneumoviral virions of the invention are thus transiently and functionally reconstituted with an externally encoded protein that is essential for infection. Preferably the externally encoded protein that is essential for infection is the attachment protein G and/or the fusion protein F, whereby the G protein is most preferred. Preferably the externally encoded protein that is essential for infection is of the same

viral subgroup (A or B) as the genome that is present in the virion. More preferably the externally encoded protein that is essential for infection is homologous to the genome that is present in the virion, whereby is meant that the protein has the same amino acid sequence as the amino acid sequence that was encoded in the genome of the virus prior to its inactivation. Alternatively, this may mean that the externally encoded protein has the same amino acid sequence as present in a wild type virion of which the amino acid sequences with one or more internally encodes proteins have 100% identity with their counter part in the virion of the invention.

In the virions of the invention, the mutation in the gene of the essential structural protein is a mutation that causes the virus produced from only the viral genome to lack the protein or to express a biologically inactivated protein. Production of virus from only the viral genome is understood to mean virus produced exclusively from the viral genome as present in the virions and in the absence of any coding sequence complementing the viral genome *in trans*. The viral genome as present in the virions is thus incapable of directing expression of the essential structural protein. This may be achieved in various ways known to the skilled person, including e.g. inactivation of the translation initiation codon, introduction of stop codons near the N-terminus of the encoded protein, one or more frame-shift mutations, deletion of one or more fragments from the gene. Preferably the gene is inactivated by deletion of at least 10, 20, 50, 75, 90 or 95% of the sequence coding for the essential structural protein. Most preferred is however, a virion in which the mutation comprises deletion of the (entire) sequence coding for the protein.

Explicitly included in the invention are virions in which more than one mutation is present. In particular, more than one viral protein-coding gene may comprise mutations that inactivate or alter the function of the protein in question, or which cause the protein to lack from the virions as described above. E.g. the cold-passaged or heat-sensitive mutations as known in the art may be combined with inactivation of the essential structural proteins as disclosed in the invention above.

Clearing of pneumoviruses like RSV from the infected host organisms requires proper cellular immunity, which will not be effectively mounted without infection of epithelial cells by the virus. However, the mutant pneumoviruses of the invention lack genetic information for a protein that is essential for infection of host cells *in vivo*. Therefore the present invention discloses methods for the production of the mutant

pneumoviruses, which include replication of mutant pneumoviruses in cells that complement (*in trans*) for the absence of the protein that is essential for infection.

In another aspect the invention thus pertains to a method for producing the above defined mutant pneumoviral virions. The method is a method for producing pneumoviral virions, whereby the virions comprise a viral genome that has a mutation in a gene coding for a protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein in a form and in an amount that is required for infectivity of the virion. The method comprises the steps of: (a) infecting a culture of a first host cell with a pneumovirus comprising a viral genome that has a mutation as defined above, whereby the host cell comprises an expression vector which directs expression, either transiently or constitutively, in the host cell of the protein in a form and in an amount that is required for infectivity of the virion; and, (b) recovery of the virions from the infected host cell culture. Recovery of virions from the infected host cell culture may include either or both recovery from the culture medium as well as recovery from the cells.

The first host cell may be any host cell in which the pneumovirus is capable of replication, with or without the simultaneous expression *in trans* of the protein that is required for infectivity of the virion. Suitable host cells for this purpose are e.g. African green monkey kidney cell cultures (such as e.g. Vero, ECACC lot 10-87, 134th passage, 1990, EMEA approved).

In a preferred method of the invention, the pneumovirus that is used to infect the culture of a first host cell culture, is produced in a method comprising the steps of: (a) providing to a second host cell one or more expression vectors which direct expression in the host cell of: (i) a viral genomic RNA that has a mutation in a gene coding for a protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity; and, (ii) a pneumoviral polymerase enzyme complex and optionally one or more further viral proteins; and, (b) culturing the second host cell whereby the virions are produced. In a preferred method, the virions produced by the second host cell are amplified by one or more further cellular infection steps employing host cells which are the same or different from the second host cell.

The second host cell may be any host cell in which the pneumovirus is capable of replication, with or without the simultaneous expression *in trans* of the protein that is required for infectivity of the virion. Suitable host cells for this purpose are e.g. African green monkey kidney cell cultures (such as e.g. Vero, ECACC lot 10-87, 134th passage, 1990, EMEA approved), or Hep-2 cells. The second host cell may be the same as or different from the first host cell.

In the methods of the invention, the viral genomic RNA is transcribed from a viral DNA copy that is under the control of a bacteriophage DNA-dependent RNA polymerase promoter and whereby the (second) host cell is provided with an expression vector which directs expression in the host cell of the bacteriophage DNA-dependent RNA polymerase. Preferably, the bacteriophage DNA-dependent RNA polymerase is a T7, T3 or SP6 polymerase.

The pneumoviral polymerase enzyme complex that is expressed from one or more expression vector(s) in the second host cell at least includes the L, P, N proteins expressed from their corresponding genes or cDNA's in the expression vector(s). For improved efficiency of viral assembly and packaging of the naked viral genomic RNA, optionally, one or more further viral proteins are expressed in the second host cells. Preferred viral proteins for this purpose include the viral matrix membrane proteins of which the M2-1 protein is particularly preferred. The L, P, N, M2-1, G or F proteins are preferably derived from the viral genome of the viral isolate which is introduced and expressed in the host cell, but alternatively also homologous proteins from other heterologous viral or non viral sources may be used.

The skilled person will appreciate that a wide variety of expression vectors and regulatory sequences (such as promoters) are available in the art for expression of the viral genomic RNA, the DNA-dependent RNA polymerase, pneumoviral polymerase enzyme complex and optional further viral proteins, as well as the essential structural protein, in the first and/or second host cells (see e.g. Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York).

For reverse genetics of RNA viruses, i.e. expression of a recombinant RNA virus such as the virions of the present invention, a cDNA copy of the viral genomic RNA is cloned into plasmids and is placed under the control of sequences that will allow synthesis of RNA from the DNA under certain conditions. Generally, the promoter

sequence for bacteriophage RNA polymerase (e.g. the T7 RNA polymerase) is placed upstream of the DNA copy of the RNA genome, while an appropriate terminator for the RNA polymerase is placed downstream of the genome. Self-cleaving ribozyme sequences are placed upstream of the terminator sequences, to allow synthesis of RNA with the correct terminal nucleotides. Correct terminal sequences are generally required to rescue virus from the synthetic RNA. For non-segmented negative strand RNA viruses, co-expression of the polymerase enzyme complex (N, P and L proteins for Paramyxoviruses) along with the genomic or anti-genomic RNA is required to obtain recombinant virus (reviewed by Neumann 2002 and exemplified in the Examples herein).

Other preferred methods may comprise the further step of isolating and/or purifying the virions of the invention and/or formulating these virions into pharmaceutical compositions. Methods for isolating and/or purifying virions are well known to the skilled virologist. Such methods e.g. include various centrifugation techniques (e.g. differential or density centrifugation), or chromatographic techniques. A method for formulating the virions of the invention into a pharmaceutical composition at least comprises the step of mixing the virions with a pharmaceutically acceptable carrier as defined below.

In a further aspect the invention relates to a composition comprising a virion as defined above or obtainable in a method as defined above, and a pharmaceutically acceptable carrier. The composition preferably is a pharmaceutical composition that is preferably suitable for use as a vaccine, i.e. the composition preferably is a vaccine.

In a yet another aspect the invention provides for a pharmaceutical preparation comprising as active ingredient a virion according to the invention, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable stabilising agents, osmotic agents, buffering agents, dispersing agents, and the like may also be incorporated into the pharmaceutical compositions. The preferred form depends on the intended mode of administration and therapeutic application. The pharmaceutical carrier can be any compatible, non-toxic substance suitable to deliver the reconstituted viral membranes to the patient. Pharmaceutically acceptable carriers for intranasal delivery are exemplified by water, buffered saline solutions, glycerin, polysorbate 20, cremophor EL, and an aqueous mixture of caprylic/capric glyceride, and may be buffered to provide a neutral pH environment.

For administration by inhalation, the pharmaceutical compositions of the present invention are conveniently delivered in the form of an aerosol spray from pressurised packs or a nebuliser, wherein the virions are present in a carrier as described for intranasal delivery but with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount.

Methods for preparing intranasal or inhalant compositions are well known in the art and described in more detail in various sources, including, for example, Remington's Pharmaceutical Science (15th ed., Mack Publishing, Easton, PA, 1980) (incorporated by reference in its entirety for all purposes). The virions may thus be formulated as the active component in any preparation for vaccination, which may further e.g. include carriers, adjuvants, stabilisers, solubilisers, preservatives and other excipients known in the art, to allow or to aid efficient administration of the preparation for vaccination to individuals, preferably human and live stock or farm animals (such as cows, pigs, horses, goats, sheep).

In a further aspect, the invention relates to a method for vaccination against, or for prophylaxis or therapy (prevention or treatment) of an pneumoviral infection by administration of a therapeutically or prophylactically effective amount of (a pharmaceutical composition comprising) the virions of the invention as defined above, or obtainable as defined above, to a subject in need of prophylaxis or therapy. Preferably, the virions are administered intranasally.

The invention similarly relates to virions of the invention as defined above, or obtainable as defined above, for use as a medicament, preferably a medicament for vaccination against, or for prophylaxis or therapy of a pneumoviral infection. The invention further relates to the use of the virions of the invention in the manufacture of a medicament for vaccination against, or for prophylaxis or therapy of a pneumoviral disease or infection. Preferably the medicament is a preparation for intranasal administration.

The compositions comprising the virions of the invention for vaccination are preferably administered intranasally to appropriate hosts. In one embodiment, calves are to be protected from b-RSV infections. In yet another embodiment, humans, of which preferably infants and elderly or immune compromised individuals are protected

from h-RSV infections. Formulations preferably comprise formulations suitable for administration as intranasal drops or spray, preferably a nasal spray. The ΔG+G- pneumoviral particles in the composition will infect epithelial cells of the upper respiratory tract only once because the second generation virions produced from the initially infected URT epithelial cells lack the G attachment protein for which the coding sequence has been removed from the genome. These ΔG-virions are therefore non-infectious *in vivo* in host organisms. However, the initial single cycle of infection allows for the development of appropriate cellular immunity - that is a response capable of clearing wild-type virus infection - to be mounted against pneumovirus, or RSV in particular, while protective antibodies against F - i.e. antibodies that will prevent lower respiratory tract infection - will be elicited by the vaccine and the non-infectious progeny. Anti-F antibodies are effective in limiting RSV infection, as is shown by the effectiveness of Palivimuzab treatment, which is a humanised monoclonal antibody against F. This is the basis of the efficacy of the recombinant live attenuated pneumoviral vaccines of the invention. These live viral vaccines solves a number of problems associated with current pneumovirus vaccine candidates. The presence of the G-protein in its natural context in the virion allows for the development of appropriate cellular immunity whereas the undesirable effects of immunity against the isolated G protein that is largely responsible for immune enhancement of b-RSV and h-RSV pathology in cattle and humans respectively, is avoided.

Description of the figures

Figure 1: Diagram of construction of pRSVXΔG. Upper line represents RSV isolate X genomic RNA, with genes indicated. Boxes below represent RT-PCR products and oligonucleotide duplexes used for construction. Numbers inside boxes indicate the oligonucleotide numbers as listed in table I. Restriction sites introduced for cloning are indicated. The final cloning scheme is indicated below: circles are plasmids and the arrows show the order of cloning.

Figure 2. Alignments showing the differences between RSV isolate X and pRSVXΔG sequences. Sequences are shown as alignment of genomic sense. For pRSVXΔG only nucleotides differing from RSV isolate X are indicated. Similar sequences are indicated by dots (.) and gaps are indicated by (-). Gene start signals are single underlined, gene stop signals double underlined, and the genes are indicated in the captions. Boxes

outline the restriction enzyme recognition sites resulting from the nucleotide changes introduced.

Figure 3. Identification of sequence markers in RSV RT-PCR amplification products, digestions digests: a) MluI, b) XmaI, c) SexA-I, d) SnaB-I.

Figure 4. Growth curves of RSV isolate X and ΔG-RSV isolate X. Vero (solid lines) and Hep-2 (dashed lines) cells were infected with virus at MOI=0.1 and incubated at 37°C. At the indicated time points cells were harvested and CCID50 titres were determined on Vero cells.

Table I. Primers used for RT-PCR cloning of RSV isolate X.

Table II. Primers used for cloning of helper plasmids and for plasmids used for construction of stable cell lines.

Table III. Primers used for diagnostic RT-PCR on RNA from RSV infected Vero cells.

Table IV. Results cotton rat immunization experiments, protection against RSV infection and RSV-induced pathology by ΔG-RSV isolate X immunization.

Examples

The current invention is illustrated by the following non limiting examples that are merely used to illustrate specific embodiments of the invention and should not be read as limiting the general scope or any aspect of the invention.

Example 1

Viral isolate, virus isolation, propagation and storage

The basis for the recombinant h-RSV clone is a clinical RSV isolate, obtained from the Leiden University Medical Centre diagnostic laboratory. This virus, named 98-25147-X, coded after the patient from which it was isolated, was derived from a diagnostic test on Hep-2 cells in the period 21-24 December 1998. It was later determined to be a subtype A isolate and is designated RSV isolate X. The virus was passaged 4 times on Hep-2 cells in T75 bottles in DMEM (Gibco), 10% FCS, pen/strep/glu and subsequently five times on Vero cells in T75 bottles on in DMEM (Gibco), 10% FCS, pen/strep/glu. The resulting RSV isolate X virus was used as working stock and stored at -135^{0}C in 25% or 45% sucrose.

Example 2

Construction of RSV-X cDNA encoding viral genome

Total RNA was obtained by phenol-guanidine isothiocyanate extraction (Trizol, Invitrogen) of stock RSV isolate X infected Vero cells. cDNA was prepared by reverse transcription using Thermoscript (Invitrogen) reverse transcriptase using random hexamer primers. This cDNA was used as template for PCR using High fidelity Taq polymerase (Invitrogen) using specific primers containing restriction enzyme recognition sites (Table I and sequence listing). Primers were designed based on the published sequences of RSV-A2 (Genbank accession no M74568) and RSV-RSS2 (Genbank accession no U39662).

PCR products were first cloned individually in different vectors: primer pairs, vectors, restriction enzyme recognition sites and resulting vector name are listed below.

RSV021/RSV047: pCAP vector (Roche), bluntly into Mlu N1, pCAP3 (SH/M/P region)

RSV018/019: pCAP vector, bluntly into Mlu N1, pCAP2 (G region)

RSV016/RSV017: PUC21, Mlu I /Bam HI, pUK5 (M2-2/M2-1/F region)

RSV024/RSV025a : PUC21, Bam HI/Afl II, pUK1 (NS2/NS1 region)

RSV022/ RSV023: PUC21, EcoR V, pUK4 (N region)

RSV014/ RSV015: PUC21, Kpn I/Mlu I, pUK2 (L region)

At least two individual clones derived from two independent cDNA templates were sequenced; regions containing differences between the two clones were sequenced on a third clone. If necessary, clones were repaired using standard molecular biology techniques known by the skilled person. Additional PCR products covering the binding sites of the primers used for cloning were obtained and sequenced. The 5' genomic termini were determined by poly-adenylation of genomic RNA, followed by RT-PCR with an oligo(d)T containing primer ALG018:

TTAAAAGCTTTTTTTTTTTTTTTTTT

and an NS1 gene primer RSV126:

AATTCTGCAGGCCCATCTCTAACCAAAGGAGT.

This fragment was cloned into pUC21 using Hind III/Pst I. The 3'-end was determined by RACE (rapid amplification of cDNA ends) ligation PCR. All sequences were assembled to yield the RSV-X consensus sequence (Seq ID No. 1).

All sequences were confirmed by PCR cycle sequencing using the BigDye terminator kit (Applied Biosystems) and analysed by an ABI Prism 310 genetic analyser.

Example 3

Construction of ΔG-RSV isolate X full length plasmid

The full-length cDNA spanning the entire RSV isolate X genome was assembled by sequential ligation of PCR fragments (Figure 1). The "trailer" end is preceded by the promoter for the bacteriophage T7 polymerase. To generate correct 3' ends the cDNA "leader" end is fused to the hepatitis delta virus ribozyme (HDVR), followed by a terminator of the T7 RNA polymerase transcription (see Figure 1).

First, two sets of complementary oligomers encoding the HDVR and the T7 terminator RSV026/RSV027 oligo's and RSV028/029 oligo's were phosphorylated with T4 DNA kinase, hybridised and ligated into clone pUK1 (containing genes NS1/NS2) via Rsr II /Not I, giving plasmid pUK3. Then, the Xma I/SexA I fragment of clone pUK4 containing N was ligated into plasmid pUK3 via Xma I/SexA I. This plasmid (pUK6) contains the region from the N gene up to the 3' leader sequence, fused to the HDVR and a T7 terminator.

Secondly, the Xma I/Eco RV fragment of plasmid pCAP3 was inserted in plasmid pUK5 using Xma I and a filled-in Hind III site. This yields plasmid pUK8. Subsequently, pUK 8 was digested with BssH II and BsiW I, ends were filled-in with Klenow polymerase and religated. This plasmid contains the genes M2-2, M2-1, F, SH, M and P and is named pUK9.

To synthesise a low-copy number vector for the RSV isolate X cDNA, two complementary oligomers, RSV011: AGCTTGCGGCCGCGTCGACCCGGGACGCGTCGATCGGGTACCAT and RSV012: CGATGGTACCCGATCGACGCGTCCCGGGTCGACGCGGCCGCA were phosphorylated with T4 DNA kinase, hybridised and inserted in the alkaline phosphatase treated and Cla I /Hind III digested plasmid pACYC184 (New England Biolabs). The resulting plasmid is named pACYC184-MCS. Subsequently a Mlu I-Knp I fragment of pUK2 containing the T7 promoter and L gene was inserted, this intermediate plasmid is named pACYC1. Then, the region from the N gene up to the 3'-leader sequence, including the fused HDVR and T7 terminator sequence of pUK6

was added to pACYC1 using Xma I/Not I. This gives intermediate plasmid pACYC2. Finally, the Xma I /Mlu I fragment of pUK9 containing the M2-2, M2-1, F, SH, M and P genes was inserted into pACYC2, yielding plasmid pACYC3, comprising the whole RSV genome of strain X lacking the G gene. Sequence analysis of the latter plasmid revealed a deletion in the HDVR region, which was repaired and the resulting plasmid is named pRSVXΔG.

In addition to construct pRSVXΔG, construct pACYC24 was generated in which the genomic RSV isolate X insert is reverse complemented via inverse PCR. From the construct, antigenomic RSV RNA can be synthesised. In pACYC24, the T7 promoter precedes the 3'-leader sequence, whereas the HDVR and T7 terminator are fused to the 5'-trailer sequence.

All restriction enzyme recognition sites used to construct pRSVXΔG are located inside the RSV intergenic regions and do not alter coding sequences or affect transcription signals (as shown in Figure 2).

Example 4

Construction of helper plasmids

Helper plasmids expressing several RSV proteins were constructed as follows. All required genes are derived from lab-strain RSV-A2 (ATCC #VR1302). Virus was plaque-purified on Hep-2 cells and subsequently used to infect Vero cells. Total RNA was isolated from these cells by phenol-guanidine isothiocyanate extraction (Trizol, Invitrogen) and subjected to RT-PCR using High Fidelity Taq polymerase (Invitrogen) and a set of primers specific for RSV genes L, P, N and M2-1 respectively (see Table II). PCR products were subsequently cloned into expression plasmids pcDNA3, pcDNA6 or pCI, using restriction enzyme recognition sites as indicated in the table II. Clone sequences were confirmed by PCR cycle sequencing using the BigDye terminator kit (Applied Biosystems) and analysed by an ABI Prism 310 genetic analyser.

Example 5

Construction of G-producing Vero cell-lines

Cell lines producing RSV-G protein were constructed using several methods:

In method 1, the G gene from either RSV-A2 or RSV isolate X, or the G gene from RSV-A2, in which the internal translation initiation codon had been disabled by modification using primers RSV033 and RSV 034, were cloned into expression vector pcDNA3 or pcDNA6 (Invitrogen) using RT-PCR on RNA from RSV-A2 or RSV isolate X infected Vero cells using primers as indicated in Table II. The plasmids were introduced into Vero cells using either chemical agents $CaCl_2$, co-precipitation, liposome-based or electroporation (Ausubel 1989). Two methods for isolating stable cell lines were used. In the first method, 72 hours after transfection, cells were split using various dilutions into fresh medium containing selective medium, zeocin for pcDNA3 and blasticidin for pcDNA6. Cells were fed with selective medium every 3-4 days until cell foci were identified. Single colonies were picked and transferred in to 96-well plate, or seeded in various dilutions to obtain single cells in a 96 well plate. Antibiotic resistant colonies were tested on expression of RSV-G by immunostaining techniques or FACS using RSV G-specific antibodies. Colonies expressing G were passaged, and were designated as stable cell lines expressing G. The second method comprises FACS sorting using RSV-G specific antibodies 72 hours after transfection. RSV-G expressing cells were seeded in a serial dilution to obtain single cells in a 96-well plate and cultured with selective medium. Single cell colonies were passaged on selective medium and subsequently tested again for expression of RSV-G, resulting in cell lines expressing RSV-G.

In method 2, the Flp-In system (Invitrogen) is used to produce Vero cells with target gene insertion sites at chromosomal positions which allow different levels of target gene expression. The RSV-G gene, derived from the plasmids from method 1 but with a modification (introduced using primer RSV151: Table II) of the G translation initiation codon surrounding sequence to allow higher translation levels, were inserted in each of these cell lines using the system-generic method, resulting in Vero cell lines stably expressing different levels of G protein.

In method 3, Vero cells were transiently made to express the G protein, by either transfection with the expression plasmids containing the G gene from method 1, or by infection with Modified vaccinia virus Ankara (MVA) (Sutter 1992) or fowlpox viruses (Spehner 1990) expressing the G protein.

Example 6

Construction of bacteriophage T7-polymerase-producing cell lines

The bacteriophage T7 polymerase gene is PCR amplified from plasmid pPRT7 (van Gennip 1997), containing the gene, using primers ALG022 and ALG023 (Table II). The PCR product is cloned into pcDNA6b vector, using Hind III/Xba I, yielding plasmid pc6T7pol. Vero cells were transfected using lipofectamine 2000 as recommended by the manufacturer (Invitrogen). 72 hours after transfection cells were split and grown in fresh medium containing blasticidin. Cells were fed fresh medium every 3-4 days and split twice to obtain larger culture volumes. 20 days after transfection, blasticidin resistant cells were transfected with reporter plasmid pT7-IRES2-EGFP using lipofectamine 2000. For the construction of plasmid pT7-IRES2-EGFP, first plasmid pT7-EGFP was constructed by inserting via HindIII/BamH1 in plasmid p-EGFP-N1 (Clonetech) a set of complementary oligomers encoding for the T7 promoter sequence (ALG32: AGCTAATACGACTCACTATAGGGAGACGCGT and ALG33: GATCACGCGTCTCCCTATAGTGAGTCGTATT). Plasmid pT7-IRES2-EGFP was then constructed by cloning the T7-EGFP fragment of plasmid pT7-EGFP into plasmid p-IRES2-EGFP via Xma1-Not1. Cells expressing EGFP were sorted by FACS and grown in limited dilution to obtain single cell colonies. Single colonies expressing T7 RNA polymerase were tested for stability, grown to larger culture volumes and stored.

Example 7

Method to produce recombinant ΔG-RSV isolate X virus

Hep-2 cells were cultivated in DMEM + 10% FCS (foetal calf serum) + penicillin/ streptomycin/ glutamine, whereas Vero cells and derivatives thereof are cultivated in M199 + 5% FCS + pen/strep/glu. Cells were grown overnight to 80% confluence in 10 mm^2 dishes at 37^0C. For Vero and Hep-2 cells, cells were infected with modified virus Ankara-T7 (MVA-T7)(Sutter 1992, Wyatt 1995) or fowlpox-T7 virus (Britton 1996) at MOI = 3 (multiplicity of infection 3) and incubated at 32^0C for 60 min prior to transfection, to allow expression of bacteriophage T7 polymerase. The cells (Hep-2, Vero or Vero-T7 cells) were washed with Optimem medium (Optimem 1 with glutamax, Invitrogen) and subsequently transfected with helper plasmids encoding the N, P, L and M2.1 genes of RSV and with plasmid pRSVXΔG, using Lipofectamine2000 (Invitrogen) in Optimem (total volume 500 μl). The following

amounts of plasmids were added: 1.6 μg pRSVXΔG, 1.6 μg pcDNA6-A2-N, 1.2 μg pcDNA3-P, 0.4 μg pcDNA6-A2-L, 0.8 μg pcDNA6-A2-M2.1. After 3-4 hrs of incubation at 32^{0}C, 500 μl of Optimem medium with 2% FCS was added and the cells were incubated at 32^0 C for 3 days. Cells were then scraped and the mixture of scraped cells and medium containing the rescued virus was used to infect fresh cultures of Vero or Hep-2 cells grown in DMEM + 2% FCS + pen/strep/glu. The latter procedure was repeated for 4-5 times to obtain high titre virus stocks.

Identity of ΔG-RSV isolate X virus was confirmed by RT-PCR on RNA isolated from ΔG-RSV isolate X infected Vero cells and digestion of the obtained products with the unique restriction enzymes whose recognition sites were introduced into pRSVXΔG (figure 2). RSV isolate X was used as control.

For the identification of sequence markers in RSV, Vero cells were infected with RSV isolate X or with ΔG-RSV isolate X with an MOI= 0.1. 72 hrs after infection, RNA from culture supernatants was isolated and used as template for RT-PCR. Primers were designed to flank the inserted sequence markers in the recombinant ΔG-RSV isolate X virus. After RT-PCR, the obtained products were digested with the appropriate restriction enzymes. The following digestion products were obtained (figure 3):

a) PCR with primer RSV065 (GTCCATTGTTGGATTTAATC) and RSV093 (CAAGATAAGAGTGTACAATACTGTC) and digestion with Mlu-I yielded the expected fragments of 937 bp for RSV isolate X, and 459 and 478 bp for ΔG-RSV isolate X

b) PCR with primers RSV105 (GTTGGATTGAGAGACACTT) and RSV113 (AGTATTAGGCAATGCTGC) followed by digestion with Xma-I yielded the expected fragments of 880 bp for RSV isolate X, and 656 and 224 bp for ΔG-RSV isolate X

c) PCR with primers RSV112 (CCCAGTGAATTTATGATTAG) and RSV160 (AATTGGATCCATGGACACAACCCACAATGA) and digestion with SexA-I yielded the expected fragments of 694 bp for RSV isolate X, and 492 and 202 bp for ΔG-RSV isolate X

d) PCR with primers RSV098 (TGGTAGTTCTCTTCTGGCTCG) and RSV114 (ATCCCCAAGTCATTGTTCA) followed by digestion with SnaB-I yielded the

expected fragments of 1820 bp for RSV isolate X, and 507 and 387 bp for ΔG-RSV isolate X.

Growth characteristics of ΔG-RSV isolate X compared to RSV isolate X were determined on Vero and on Hep-2 cells (figure 4).

Table III. Primers used for diagnostic RT-PCR on RNA from RSV infected Vero cells.

Primer name	*Sequence*
RSV065	GTCCATTGTTGGATTTAATC
RSV093	CAAGATAAGAGTGTACAATACTGTC
RSV098	TGGTAGTTCTCTTCTGGCTCG
RSV105	GTTGGATTGAGAGACACTT
RSV112	CCCAGTGAATTTATGATTAG
RSV113	AGTATTAGGCAATGCTGC
RSV114	ATCCCCAAGTCATTGTTCA
RSV160	AATTGGATCCATGGACACAACCCACAATGA

Example 8

Method to produce recombinant ΔG+G-RSV isolate X virus

ΔG-RSV isolate X virus, derived from transfected Vero cells, was passaged several times to obtain titres of at least 10^5 pfu/ml (plaque forming units per ml). Different moi's of this virus were then used to infect the Vero cell line producing RSV-G protein. The resulting ΔG+G-RSV isolate X was harvested from the medium and/or from the cells and analysed for the presence of the G protein in the virions by immunodetection techniques. Infectivity titres were determined on Vero or Hep-2 cells, and the integrity of the ΔG-genome was determined using RT-PCR on viral RNA extracted from cells infected with ΔG+G-RSV isolate X virus. Virus was stored at -135^0 C in 25% or 40% sucrose.

Example 9

Method to protect in a cotton rat animal model against RSV infection and RSV-induced pathology by ΔG-RSV isolate X immunization

Protection experiments were performed in cotton rats (Sigmodon hispidus, 5-6 weeks old, 4-6 animals per group and both sexes). In initial experiments, this animal was shown to be sensitive to RSV infection and to exhibit severe vaccine-mediated lung pathology as described by Prince, 2001 and which closely mimics the human situation. After intranasal application of RSV lung pathology was characterized by inflammation infiltrate in and around bronchus/bronchioli and hyperplasia of epithelium. A more severe pathology was seen upon intramuscular immunization with formalin-inactivated RSV-A2 followed by an intranasal challenge with RSV-A2. In addition to the above-mentioned pathology, perivascular and peribronchiolar infiltrate and alveolitis were observed, characteristic for an immune-mediated pathology. These observations were used as "internal" reference for all immunization and challenge experiments. Infection and immunization of cotton rats with RSV preparations was done intranasally, in both nostrils. Cotton rat lungs were examined for pathology lightmicroscopically and virus titres at different time points post-challenge or post-infection/immunization were determined on Vero cells using serial dilutions of lung homogenates with RSV specific ELISA to yield $CCID_{50}$ titres and immunostaining using RSV specific abs to yield pfu titres. After immunization twice with ΔG-RSV isolate X cotton rats were fully protected against infection and pathology caused by RSV isolate X in the lungs. The results from several experiments are summarized in Table IV.

Table IV:

			infection with:	*t*[1]	*V*[2]	*lung pathology day 5 post infection*	*lung t*[3]
			ΔG-RSV isolate X	5	100	yes, moderate	below detection
			RSV-A2	5	100	yes, strong	2*5
			RSV isolate X	5	100	yes, strong	4*5
immunization day 0 and 21	*t*[1]	*V*[2]	*challenge day 42*	*t*[1]	*V*[2]	*lung pathology day 5 post challenge*	*lung t*[3]
2x ΔG-RSV	5	100	RSV isolate X	5	100	no	below

isolate X						detection
mock	100	RSV isolate X	5	100	yes, strong	5

[1]: virus titres in logs pfu/ml
[2]: volume in μl per animal, which is half this volume in each nostril
[3]: virus titres in logs per gram lung, detection limit is 10^2 $CCID_{50}$

References

Alwan WH, Record FM, Openshaw PJ. Phenotypic and functional characterisation of T cell lines specific for individual respiratory syncytial virus proteins. J Immunol. 1993, 150(12):5211-8.

F.M. Ausubel, R. Brent, R. E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1997

Bembridge GP, Rodriguez N, Garcia-Beato R, Nicolson C, Melero JA, Taylor G. DNA encoding the attachment (G) or fusion (F) protein of respiratory syncytial virus induces protection in the absence of pulmonary inflammation. J Gen Virol. 2000, 81(Pt 10):2519-23.

Britton P, Green P, Kottier S, Mawditt KL, Penzes Z, Cavanagh D, Skinner MA. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. J Gen Virol. 1996, 77 (Pt 5):963-7.

Buchholz UJ, Granzow H, Schuldt K, Whitehead SS, Murphy BR, Collins PL. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory syncytial virus (HRSV): effects on host range and evaluation as a live-attenuated HRSV vaccine. J Virol. 2000, 74(3):1187-99.

Cane PA, Matthews DA, Pringle CR. Identification of variable domains of the attachment (G) protein of subgroup A respiratory syncytial viruses. J Gen Virol. 1991, 72 (Pt 9):2091-6.

Collins PL, Purcell RH, London WT, Lawrence LA, Chanock RM, Murphy BR. Evaluation in chimpanzees of vaccinia virus recombinants that express the surface glycoproteins of human respiratory syncytial virus. Vaccine. 1990, 8(2):164-8

Crowe JE Jr. Immune responses of infants to infection with respiratory viruses and live attenuated respiratory virus candidate vaccines. Vaccine. 1998, 16(14-15):1423-32. Review.

Gonzalez IM, Karron RA, Eichelberger M, Walsh EE, Delagarza VW, Bennett R, Chanock RM, Murphy BR, Clements-Mann ML, Falsey AR. Evaluation of the live attenuated cpts 248/404 RSV vaccine in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults. Vaccine. 2000, 18(17):1763-72.

Greenough A, Thomas M. Respiratory syncytial virus prevention: past and present strategies. Expert Opin Pharmacother. 2000, 1(6):1195-201.

Jin H, Cheng X, Traina-Dorge VL, Park HJ, Zhou H, Soike K, Kemble G. Evaluation of recombinant respiratory syncytial virus gene deletion mutants in African green monkeys for their potential as live attenuated vaccine candidates. Vaccine. 2003, 21(25-26):3647-52.

Karron RA, Buonagurio DA, Georgiu AF, Whitehead SS, Adamus JE, Clements-Mann ML, Harris DO, Randolph VB, Udem SA, Murphy BR, Sidhu MS. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterisation of a cold-passaged, attenuated RSV subgroup B mutant. Proc Natl Acad Sci U S A. 1997, 94(25):13961-6.

Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol. 1969, 89(4):422-34.

Li X, Sambhara S, Li CX, Ewasyshyn M, Parrington M, Caterini J, James O, Cates G, Du RP, Klein M. Protection against respiratory syncytial virus infection by DNA immunization. J Exp Med. 1998, 188(4):681-8.

Lofland JH, O'Connor JP, Chatterton ML, Moxey ED, Paddock LE, Nash DB, Desai SA. Palivizumab for respiratory syncytial virus prophylaxis in high-risk infants: a cost-effectiveness analysis. Clin Ther. 2000, 22(11):1357-69.

Mufson MA, Belshe RB, Orvell C, Norrby E. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J Infect Dis. 1988, 157(1):143-8.

Neumann G, Whitt MA, Kawaoka Y. A decade after the generation of a negative-sense RNA virus from cloned cDNA - what have we learned? J Gen Virol. 2002, 83(Pt 11):2635-62. Review.

Openshaw PJ, Dean GS, Culley FJ. Links between respiratory syncytial virus bronchiolitis and childhood asthma: clinical and research approaches. Pediatr Infect Dis J. 2003, 22(2 Suppl):S58-64; discussion S64-5. Review.

Peebles RS Jr, Hashimoto K, Graham BS. The complex relationship between respiratory syncytial virus and allergy in lung disease. Viral Immunol. 2003;16(1):25-34. Review.

Plotnicky H, Siegrist CA, Aubry JP, Bonnefoy JY, Corvaia N, Nguyen TN, Power UF. Enhanced pulmonary immunopathology following neonatal priming with formalin-inactivated respiratory syncytial virus but not with the BBG2NA vaccine candidate. Vaccine. 2003, 21(19-20):2651-60.

Power UF, Plotnicky-Gilquin H, Huss T, Robert A, Trudel M, Stahl S, Uhlen M, Nguyen TN, Binz H. Induction of protective immunity in rodents by vaccination with a prokaryotically expressed recombinant fusion protein containing a respiratory syncytial virus G protein fragment. Virology. 1997, 230(2):155-66.

Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. J Gen Virol. 2001, 82:2881-8.

Robinson RF, Nahata MC. Respiratory syncytial virus (RSV) immune globulin and palivizumab for prevention of RSV infection. Am J Health Syst Pharm. 2000, 57(3):259-64. Review. Erratum in: Am J Health Syst Pharm 2000 Apr 1;57(7):699.

Sambrook J., Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989

Schmidt U, Beyer J, Polster U, Gershwin LJ, Buchholz UJ. Mucosal immunization with live recombinant bovine respiratory syncytial virus (BRSV) and recombinant BRSV lacking the envelope glycoprotein G protects against challenge with wild-type BRSV. J Virol. 2002, 76(23):12355-9.

Siegrist CA, Plotnicky-Gilquin H, Cordova M, Berney M, Bonnefoy JY, Nguyen TN, Lambert PH, Power UF. Protective efficacy against respiratory syncytial virus following murine neonatal immunization with BBG2Na vaccine: influence of adjuvants and maternal antibodies. J Infect Dis. 1999, 179(6):1326-33.

Spehner D, Drillien R, Lecocq JP. Construction of fowlpox virus vectors with intergenic insertions: expression of the beta-galactosidase gene and the measles virus fusion gene. J Virol. 1990, 64(2):527-33.

Srikiatkhachorn A, Braciale TJ. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia

during experimental murine respiratory syncytial virus infection. J Exp Med. 1997a, 186(3):421-32.

Srikiatkhachorn A, Braciale TJ. Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection. J Virol. 1997b, 71(1):678-85.

Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci U S A. 1992, 89(22):10847-51.

Van Gennip HG, van Rijn PA, Widjojoatmodjo MN, Moormann RJ. Recovery of infectious classical swine fever virus (CSFV) from full-length genomic cDNA clones by a swine kidney cell line expressing bacteriophage T7 RNA polymerase. J Virol Methods. 1999, 78(1-2):117-28.

Wyatt LS, Moss B, Rozenblatt S. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology. 1995, 210(1):202-5.

SEQUENCE LISTING

<110> NVI Nederlands Vaccin Instituut
<120> A Respiratory Syncytial Virus with a genomic deficiency complemented in trans
<130> P210823 pct
<160> 33
<170> PatentIn version 3.1

<210> 1
<211> 15213
<212> DNA
<213> Human respiratory syncytial virus

<400> 1
acgagaaaaa aagtgtcaaa aactaatatc tcgtagttta gttaatatac atataaacca 60

attagatttg ggtttaaatt tattcctcct agatcaaaat gataatttta ggattagttc 120

actagaagtt attaaaaatt atataattat taattttaaa taactataat tgaatacagt 180

gttagtgtgt agccatggga atttttatta taagattttt gttcattatt cattatggaa 240

gttgtataac aaactacctg tgattttaat cagtttttta agttcattgg ttgtcaagct 300

gtttaacaat tcacttagat gaggatatgt agattctacc atatataaat gattatagtt 360

taattctgtt gatctgaaat ttaaaacatg attgaaccac tttaagatgt tcatgtgctt 420

atgatttata agtttattgc tgaaaacttc attacgtcca gctatagaat aagatagtat 480

atctccacta acaacactct ttagtttaga caatgcagta ttaattcctt tttttgttat 540

agggtaacaa agaaagggta tcaaactctt aatatttgca tcaatagact ctttatcagc 600

cttcttaggc atgatgaaat ttttggttct tgatagtatc aatttagcat tttgtactac 660

attaaatact gggaacacat ttgcaggacc tattgtaagg actaagtaaa cttcagatcc 720

ctttaactta ctgcctaagc atacataagt ttttaatata gttatgttgt ctaatttgaa 780

atcgatatca tcttgagcat gatattttac tattaacgta catttattaa ctgaagaaca 840

gtacttgcat tttcttacat gcttgctcca ctctattata attttattcc agttgactgt 900

tacaggcaat tcagcatcac agacaaaaag gctgataggt tcagcaaact ttatatgtaa 960

ataagaccaa tgaatgttgt tggttgcatc tgtagcagga atggtcaaat tttcaccata 1020

atcaatgttg atatgtccat tgtacagcct taaaaactca attggtaaac tatgatcatt 1080

acaatctttc agacttctgt aaatatatct tatatcagga tgaagttcca ctactgtacg 1140

caataataaa ttccctgctc cttcacctat gaatgctata caattaggat ctttaatttt 1200

aaggtctttt aaaatatact ctatactaat tttacaacct gtagaactaa atacaaaatt 1260

gaatctatta atatgatgcc aaggaagcat gcaataaagt gatgtgctat tgtgtactaa 1320

agatatttga tgagaagtag tagtgtaaag ttggttagat ttggctgtat tacctgaatg 1380

atctataatt ttatcaatca caaccgtagg aaataaatta tacaaatctt gtctgctgta 1440

attggttcta atcattgtag acgatttaat aagcttctta ttagataaca atggtaacat 1500

tattgagtca acatttttac ctatacaata gtcattcagt gtctttttat cattactttt 1560

aaccggattg gttagtatat tttctagggt ttctggtgta ggatgatata atttgttgta 1620

attactttct aattcagaat tagcaatcct tatatgttta gttaatagat gagtattatc 1680

tgagaagtta taattaatgt aaaaaagatt agaagtataa aattcatcat tgaatttgtg 1740

tttatttta atgtatattc tatctatatt tatcaatccc attctaacaa gatctatata 1800

agttaatatt gctttcatat gtgttggatg ataatctatg ttaacaaccc aagggcaaac 1860

tgtgaattct gctacattaa gacgtttaag aaaccatagt ttgaagctat gacatccttt 1920

tactctatgt aaacttgcat cctggctaag aatgtatttg ataacttttt gttctaaaaa 1980

taccttagac atagacttcc aataactact gtctattaat tccaatacac ataggagatc 2040

tgaagtattc atatcacact ccagctttgc tctgctgtaa cctttatgaa aacacaagag 2100

atatgtctta taagcattga agaaaacttt caaattaatg aacatatgat cagttatata 2160

tccctctccc caatcttttt caaaaatacc tttagaatct ttcataagtt gtataatcag 2220

aatccaatgt ccagctaaat tagtacttaa aatgtaagta ttatgaaaat agtcagatat 2280

cttatgcgcc aatattaaat tagaattaac attagatcca gattttagtg ttttattact 2340

taagaataat tccacatatt gagtcaaact tattttgtct ggtaaaaaca tatgctgttt 2400

ttgtatcact tgttttaact tgtgaatatc aacatcacct gtgaatatgg gaggtttcat 2460

caaatgtatc tcattaagct tgggtatgag aataattctg ttaggacata cattagtaaa 2520

ttgttctact actgacatta agctaaggcc aaagcttata cagttttgga atactatatc 2580

aatatcttca tcaccatact tttctgttaa tatgcgatta atagggctag tatcaaagtg 2640

atagtttgta gttctatagg ctggtattga tgcagggaat tcacatggtc tactactgac 2700

tgtaagacga tgcaaatagt taacacttaa atattgtgga aataattttt tggccttctc 2760

atatgctaac ccaagagttc ctatgctaag ttcctccatg aattcatcct tgttatctat 2820

agatgcatac acccaatcca attttgctaa tagatctatt tgatctctct gtttttggt 2880

taaaacttgt ctattataaa ctggcattgt ttttttctct tgtgtagatg aaccaaccca 2940

tggtttagtg ggtcctctct caccacgtgt taaactgttg acattatatt tctctatgat 3000

tatgccacta gctatagtgc ttgttgtata tttgatgtcc attgtataca tgatactggg 3060

tgatgtaaca ccaactatat tggataaaga ccaagatctt tctctaacat atttacttaa 3120

ttcagtaata cttaggtttt ccatactcaa tatttctctt ttatctctgt tacaatctaa 3180

tggtaatatc cttataagca aagttatgtt tttcctcatc atctcagtgg ctctatcaat 3240

atctgttaag tctatggcag aagtcttttc cagtatgtta gttatagatt ttgtaccgga 3300

tataagattt actatttttct ctgctttata aaagggtaaa ctttcataaa caactcttag 3360

cccgtgagga tatgtaggtt ctatattttg cataatatca ttaagatcta tctctgtagt 3420

ggtatagtgt tgtgcacttt tggagaatat tttgtttgga gctgtgctca aaacctcagt 3480

aactgccagt ctattgattt cactagtaat tttagcttgc ctctcagatc ctaaagcttg 3540

aggatctctc atcaatgtta caaattcagc attagggttt ttgtcaaacg tgattatgca 3600

tgttaagaat ttattcaatc tatcatctga cagatcttga agtttatctt ttaaatcatg 3660

gtttgtataa taactaagta tgaacacaga gtgaactata gcctctgtga gaaaatcagg 3720

agttcttcta tagaaacttc gatataacaa gttgggatca ccaccaccaa ataacatggg 3780

caaattcata tacaatgtta atgctgtatc aatattatca agattaaaaa aggtttttaa 3840

gtgttttaga acctttaata tgtccaaata taatttgttg ttacataatg catgattttt 3900

tagttgtaaa gcaatttgat tatataacca tacatttcta aatattaaac tgcataatag 3960

actttcacct ctatattcta attcttgtgt caaactacct atagattcta gactcacttt 4020

gaaatcatca agtatagtgt ttatccacgg tcccactctt aggactttct ttatactagc 4080

tgggtaatat acaccgttat gttggatcgt tttactcata aattgcatat ctcttgatat 4140

ataagtctca gttcctttta atttgtggcc tatgcctgca tactctttat acagtaattt 4200

gagactgttt aatgctagca aataatctgc ttgagcatga gtttgacctt ccatgagtct 4260

gactggttta cttatatcta ttgattgatt gtcaccatta attaaagcag taattgagaa 4320

tttccctttg agagatatta gatctaatag tgatatagct tctatggtcc atagtttttg 4380

acaccaccct tcgataccac ccatatgata tctatataat ccactttgct catctacatt 4440

gttaagatct acaatatgat cccttatata gggggggtgca tgcctatatg tgcatattat 4500

tgtgacatga ggaatagtta aatgtaacca ggaaaataga gattgtacac catgcagttc 4560

atccagtaca tcactacaaa tacatgatgt ttcatatcga aatgcttgat tgaatttgct 4620

gagatctgtg atgatagagc acttactaat gtaattgttg taattatcat tgtaacgatt 4680

tgatttgtta cttattcctg ctttcaattc taatattttc tgtagttcta gatcaccata 4740

tcttgtaaga ctttcaggga aaaattgtaa aatgttttca gctatcattt tctctgctaa 4800

tatttgaact tgtctgaaca ttcctggttg cattgcaaac attctaccta cactgagttc 4860

tctttcttta cctgtcaatg ataccacatg attagggttg ttaagataac tttgattaac 4920

tacacagttg tataaatcac attcattgaa tttgttatct cttaaatagt actctaatac 4980

tcttcttgat ttatcactct cagagaattt taacttttca tgttctatat aattttgtat 5040

gtgtgacggc atataatttc tagggaaact agtccatatt aaatttttag gaggtgatat 5100

agccttatca tttatgatca tttcgagatc cacttttta ggcaatcgaa actcccgata 5160

gaaacgtagt cctgatagaa caatcaaatc tctttctgta agttccaaca aggaaggata 5220

agtgtttagt ttatagtaag ttaaccatct taagggtaaa acaatggcat tccttaaagt 5280

aggccatctg ttgtaattat ttacaaaccc ttttataatt ctatatataa aggcacctct 5340

taacatactc aaactgctta acaagtaaaa tttggtctcg ttgcaattaa ctttaacagc 5400

atccatggct tgtctttcat ctaccattgg gtgtccaaat attctgaaca aaaaatataa 5460

ttcactcaga ttgttaaggt tattgtcacc tgcaagctta attaatttta ggaacttact 5520

taatagaatt atccatctgc catttattat attatcggat actgtcttat ctaataatgt 5580

atgacatact cttgatagca gatttttctg agctttatta gcagcatctg tgatgttgtt 5640

gagcatacta ttataaaacc gttttctgaa ttgatcttct tctgttatat ttaaaattag 5700

agacataata aatccctcta cctcttttat tatgtagaac ccctcattgt gaaatagttt 5760

tagtatacaa tctccataaa ggaataattg tgtcaagata acattattga atccacatct 5820

taagcctaag cttttattta atgtgttcaa acagttacta atccatgtaa tcaaacaaac 5880

atttaatcta ctaaggctaa tatctttcca tgtcaagaat tgattatagg ttgtcacagt 5940

aattcttttg agttccttat gataaactat acaaccatat tgattcaaaa taaattggaa 6000

tccattaaga gtatgattat ctatcaatat aaaaccatgg ttttttacct cactagatcg 6060

atactgtgtt aatatgctgt ttaattttgt gtataaatta aaccaatgta ttaaccatga 6120

tggaggatgt tgcatcgaac acattaattt cttcaagagt gttgttttga ttgtatcttt 6180

ttgttttgta gagtgatttt tgtctgcttt aagatgagat tgattatcct taacagctaa 6240

aagtatatca tctttgatta tggttgtaat aactgagttg tcttcatctt gtccattgtt 6300

ggatttaatc ttgtcttttt ctttaagccc cagtttattc agtatagcat agactttgac 6360

atcactaatt tctatagctc ttcttattat ctttttaagt aaattagtgg tagtaatctg 6420

ttctgacgag gtcatactct tgtatgtcat aagtaatgac tgaaaatagg taggttcttc 6480

tatttttatt tcacctttat gatacttaga tattaaggac tgtgttatat ttagtttctt 6540

tagatttatg tgttctatta atggattttg tctactaatt aagttggtat aatcattttt 6600

gagataagga ccattgaata tgtaacttcc taaagcatta cattctgaga aagaaataac 6660

acctttaaa taactatcag ttagataaac attagcagaa tttccattaa taatgggatc 6720

cattttgtcc catagcttga attgtttgag ttaatagttt gatgatgtgg taagcattag 6780

gattgagtgt tatgacacta atatatatat tgtgtatata tcatcattaa tacctagatg 6840

ttgtagaaaa ttttgagttg catcaatcaa gtcttgagag gtccaatgga tttcattgaa 6900

tggttgattc ggtgagtata tatggttatt ttggttggtt tgattgatat atagtgtgtt 6960

tttttgatta tacatagtaa ctctacaact acttgttatt agtatggaat ttatactaca 7020

aggatatttg tcaggtagta tcattatttt tggcatggtc gttcgtatca ctaacagttg 7080

attcttttgg gttattgatg gttatgctct tgtggatatc caatgtgttt ttgatggttt 7140

tcttcaatac atctgccggc aatcttttta acagatgaat agtttgttta ttgtttttcc 7200

tgttgctttc aatatatgat atgacagtat tgtacactct tatcttgggt gaatttggct 7260

cttcattgtc ccttagtttt ttgatgtcat cactgttgag ttcagtgagg agtttgctca 7320

tggcaacaca tgctgattgt ttagttatat tatttattga tcctatataa ctctctagca 7380

ctccaactac accgagggca tactcttctg ttctgtccaa ctctgcagct ccacttattt 7440

ctgataaagt atctatgctt ttatccatag acttaagtat tctgtttaac ataaaatttt 7500

gtcttacaag cagtgcatgg ggtggccatt caaaataatt atgactaaaa tggcacctct 7560

tgccattcaa gcaatgacct cgaatttcaa atttgcaagg attccttcgt gacatatttg 7620

ccccagttct tatttttaca aatagtaagt taatctggta ttcaattgtt ttatataact 7680

ataaaatagg aatctactta aatagtgtaa gtgagatggt ttatagatga aagttgtgat 7740

gaagttcaaa ttttaagaaa atccaatgat agatgggtta tctatggtta gatagtgaac 7800

cattgtaaga atatgattag gtgctatttt tattcagcta ctaaatgcaa tattgtttat 7860

accactcagt tgatccttac ttagtgtgac tggtgtgctt ctggccttgc aatatagaag 7920

cagtccaact gcaattaatg ataacaatat tactataatc actataatta tagtagttat 7980

catgatattt gtggtggatt taccagcatt tacattatgt aataattcat ctgatttacg 8040

aataaatgct agactctggt taatcttctc attgacttga gatattgatg catcaaattc 8100

atcagagggg aacactaatg ggtcatagaa atttattatt ggttcacctt ttacatagag 8160

acttttgcct tcttgcttat ttacataata taatgtatta cctacagaca cagtatccac 8220

ccccttattt gatacataat cacacccgtt agaaaatgtc tttatgatcc cacgattttt 8280

attggatgct gtacatttag ttttgccata gcatgacaca atggctccta gagatgtgat 8340

aacggagctg cttacatctg tttttgaagt cataattttg caatcatatt tggggttgaa 8400

tatgtcaatg ttgcagagat ttacctcact tggtaatgtt aaactgttca ttgtatcaca 8460

aaatacccga tttgattgaa ctttacatgt ttcagcttgt gggaagaaag atactgatcc 8520

tgcattgtca cagtaccatc ctctgtcggt tcttgttaag cagatgttgg acccttcctt 8580

tgtgttggtt gtacatagtg gggatgtgtg cagtttccaa caaggtgtat ctattacacc 8640

atatagtggt aattgtacta catatgctaa gacttcctcc tttattatgg acatgataga 8700

gtaactttgc tgtctaacta tttgaacatt gttggacatt aacttttct gatcatttgt 8760

tataggcata tcattgatta atgataataa ttcactatta gttaacatat aagtgcttac 8820

aggtgtagtt acacctgcat tgacactaaa ttccctggta atctctagta gtctgttgtt 8880

cttttgttgg aattctatca cagtttcaat gtttgatatg ctgcagcttt gcttgttcac 8940

aataggtaac aactgtttat ctatatagtt tttgagatct aacactttgc tggttaagac 9000

actgactcca tttgataagc tgactacagc cttgtttgtg gatagtagag cacttttgat 9060

tttgttcact tccccttcta ggtgcaagac cttggatacg gcaatgccac tggcgattgc 9120

agatccaaca cctaacaaaa agccaagaaa tcttcttttc cttttcttgc ttaatgttac 9180

attggtgttt ttggcattgt tgagtgtata attcataaat cttggtagtt ctcttctggc 9240

tcgattgttg gctgctggtg tgctttgcat gagcaattgt aattctgtta cagcattttt 9300

atatttatct aattcttgtt ttatcaattt taccttagcg tctgttccat tacacttatt 9360

ttccttgata gtacttaatt ctatagttat aacactagta taccaaccag ttcttagagc 9420

gctaagatag cccttgctaa ctgcactgca tgttgattga tagaattctt cagtgatgtt 9480

ttgactggaa gcgaaacaga gtgtgactgc agcaaggatt gtggtaatag catttgtttt 9540

gaggattggc aactccattg ttatttgccc catagttgat tttgattctg tttgatttgg 9600

tcatggcttt ttgcaataat acgcttttta atgactactg gtttgttgtg ttggatggag 9660

atagagattg tgataggtac tcggatgttg tatagacttg tgaagggctt ggattgcctt 9720

cggaggtggt tgagtggaga gtttcctttt gacttgtgtg ttctggattt cctgtggtgt 9780

tggaggtgag cagtgtagtt ctgatgtttg ttttggtggt gttgatggtt ggcttttctg 9840

tgggcctggt ggtaagtgct tcctttggtt ttgtggtttg aggtttgaga tcttttttgg 9900

ttgtcttgat ggttggtttt cttgtgggct tggtggtggt tttctttcca ggtttttttgt 9960

ttggtattct tttgcagatg gcccagcaag ttggattgtt gctgcatatg ctgcagggta 10020

caaagttgaa cacttcaaag tgaaaatcat tatttggttt gttttgtggt ttgttttggc 10080

gttgttttgt ggtgggcttg ctgggttgta tttgggttgt tgttgtgttt ttggtcttga 10140

ctgttgtgga ttgtggggtt gacttagcac ttggtgttgt tgaagctagt gtggtggtgg 10200

gttgtgatgt agtttcggac agattggaga ggctgattcc aagctgggga ttctgggtga 10260

ggtatgttgg ggttgtgttc ttgatctggt ttgttgcatc ttgtatgatt gcagttgttg 10320

gtgtgacttt gtggtttgcc gaggctatga atatgatggc tgcaattata agtgaagttg 10380

agattatcat tgccaaaata gataatgtga tttgtgctat agatttaaga tttaacttgt 10440

ataagcacga tgatatgaat aatagatgat tgagagtgtc ccaagtcctt tctagtgtct 10500

tggcggcgcg ttggtccttg gttttggaca tgtttgcatt tgccccaatg ttgttgttgg 10560

tcttaatatt ttagttcatt gttatgacta ttttctaatt aactacttta tggtatagat 10620

gatggcttgc atggtgagac gttgatgtgg ttttgtgaag aggtgagggt agttcactta 10680

caaatgcaag gttactgttt tgagctatca gattggtgaa tgctatgtat tgactcgagc 10740

tcttggtagc tcaaaggttt tgttatggaa tatgttatat tcgcagagtt tgtttagtat 10800

tgcaatcatg atggagatta tgattagcaa agagattatt gttgttatca tgtgtattag 10860

tgtaaagtaa ggccagaatt tgcttgagaa ttctattgtt atggatgtat tttccattgg 10920

ttgattttgt ctaatgtgtt gactagtcta tgttgacaga tgttgtgatt agttggattc 10980

ctctcaatga ttatttgccc catgtggatt ttttattaac ttatttgagt actggatctg 11040

atgaacaatg acttgggatg atctgagact cctgatgagt tttgtttgat tggttgaacc 11100

acaaagggtt ggtgattacg attgtgaagt gaagaatgta ggtagaaagt ttgtatgaat 11160

caactcactg atgtagagga aaaaggttaa tcttccatgg gtttgattgc aaatcgtgta 11220

gctgtgtgct tccaatttgt tgtaacataa tatatacttt ctttttctaa gtaagctcca 11280

agatctacta tgaattgact ttgtggcttt atgtatttga atgctccttt gttgtcagtc 11340

actgtgatga ctaacagtaa tcctgagtaa gggatgattt ttgcatttgt aatagcattt 11400

ttgaattcag tggttgttat attttcaagt gtgttcagat ctttatttct gacactgatg 11460

gatctcaggt atgttggtat tatgactttt tttgatgtta ctatattttc aaattcacat 11520

aaagcaatga tgtcatgtgt tgggttgagt gttttcatag tgagatcttt aactgtagtt 11580

aacatatttt ttgattttag gcatgttaga ctgcatgcct taatttcaca gggtgtggtt 11640

acatcatatg ccagcttgct tctttcatcc aaggacacat tggcacatat ggtaaatttg 11700

ctgggcattt gcgctagcac tgcacttctt gagtttatca tgactcttaa tgatggtccc 11760

ttgggtgtgg atatttgttt cactagtata ttgacattgg ctagttcttt tataagtaaa 11820

tctgctggca tggatgattg gaacatgggc acccatattg taagtgatgc aggatcatcg 11880

tctttttcta ggacattgta ttgaacagca gctgtgtatg tggagccctc gtgaagtttg 11940

ttcacgtatg tttccatatt tgccccatct ttttttgtaa ctatagtatc gatttttttcc 12000

gggtggctag ttttggattg gctggttgtt tttttggctg gttggctaat cggcaaatgg 12060

atgtttggtt ggatgggtga attggtttgt ttgttagtct tctattgatg ttgtgttttg 12120

atgtgcagat aggtagctaa tcagaaatct tcaagtgata gatcattgtc actatcattc 12180

ccttccaaca ggttgttcaa tttttctgat gttggattga gagacacttc atctgatgtg 12240

tcttttgcca tcttttcact ttcctcattc ctgagtctcg ccatagcttc tagtctgtca 12300

ttggtcatta atgcttcagt tctgattttt tctatcattt cttctcttaa accaaccatg 12360

gcatctctta taccatcccg agcagatgta ggtcctgcac tcgctactac taatgtgtga 12420

agcattccta gtatttcact taatttctca tcaatcctat ctaatcttgc tgttatatta 12480

tcgtttgtct ggtcattaat ttcttcatat gaatagctag attcttcttc attgttatca 12540

aatgtttcta tggtttcttt gtatagtttt gaaaagggat tatcacttgg cgtagggtct 12600
tctttgaaac ttactagagg ttttctttga taattgggct tgttccctac agtatcatct 12660
gtctcattta ttgggtttat aatggttgaa tttgatgtta tagggctttc tttggttact 12720
tctatatcta ttgagttgac agatatgata ctatcttttt tcttgggatc tttgggtgat 12780
gtgaatttgc cctttattga ttctaggaat ttggtggctc tgttgtttgc atcttctcca 12840
tggaattcag gagcaaactt ttccatgatg ttttatttgc cccatttttt ttattaactc 12900
aaagctctac atcattatct tttggattaa gttgatgttt gatagcctct agttcttctg 12960
ctgtcaagtc taatacactg tagttaatca caccattttc tttgagttgt tcagcatatg 13020
cttttgcagc atcatataga tcttgattcc ttggtgtacc tctgtattct cccattatgc 13080
ctaggccagc agcattgcct aatactacac tagagaagtg aggaaattga gtcaaagata 13140
ataatgatgc ttttgggttg ttcaatatat ggtagaatcc tgcttctcca cccaattttt 13200
gggcatattc atacacctcc acaacctgtt ccatttctgc ttgcacacta gcgtgtccta 13260
acataatatt tttaactgat tttgctaaga ccccccaccg taacatcact tgccctgcac 13320
cataggcatt cataaacaat cctgcaaaga tcccttcaac tctactgcca cctctggtag 13380
aagattgtgc tataccaaaa tgaacaaaaa catctataaa gtgaggatat ttttcaaaca 13440
cttcatagaa gctgtttgct atatccttgg gtagtaagcc tttataacgt ttcatttcat 13500
tttttaagac attattagct ctcctaatca cagctgtaag accagatcta tcccctgctg 13560
ctaatttggt tattactaat gccgctatac ataatattat catcccacaa tcaggagagt 13620
catgcctgta ttctggagcc acctctccca tttcttttag catttttttg taggatttc 13680
tagattctat ctcaatgttg atttgaattt cagttgttaa gcttgctaat gttaacactt 13740
caaatttcat ttctttccca ttaatgtctt gacgatgtgt tgttacatcc actccatttg 13800
ctttaacatg atatcccgca tctttgagta tttttatggt gtcttctctt cctaatctag 13860
acatagcata taacatacct attaacccag tgaatttatg attagcatct tctgtgatta 13920
atagcatgcc acatagcttg ttgatgtgtt tctgcacatc ataattagga gtgtcaatgc 13980
tatctcctgt gctccgttgg atagtgtatt tgctggatga cagaagttga tctttgttga 14040
gtgtatcatt caacttgact ttgctaagag ccatttttgt atttgcccca tctttcatct 14100
tatgtctctc cttaatttta aattactata attttcaggc tccatttgga ctatggagtg 14160
tgattgtgca tgaagttatt atttcagatt gtttgaatct tgttttgaaa ttcatggatt 14220
gagatcatac ttgtatatta tgggtgtgtg cttagtaggc ttaatgccaa tgcattctaa 14280
gaacccatca tgattgataa atattggcat agggaaagtg ccatattttg tgttgtattc 14340
agtatatttt ttatatttag tgcttcccac tttgtgcaat agtttcattt catagttgac 14400
caggaatgta aatgtggcct gtctttcatc aagttttctc actatgcatt catgatttat 14460
caagtatata aatctatgtg ttatgatgtc tctggttagt gatgttatta tagtctcaag 14520
tgacaatggt ctcatgtcag tgatcatcag tctttgtggt gtggtatcat tgtgtgttgt 14580

gtccatggtt gggtcagctt agttgattta tttgccccat ttttatcttc tgtcaagttt 14640

tatattaact aatggtgtta gtgacattga tttgctagtt gatatttatt ataatttatg 14700

gattaaggtc aaatccaagt aattcagata attgattcat ataattggtc attgttgaat 14760

cacttagttt tttggagaat ttaatttcac aattgtcatc tattaggcca ttaggttgag 14820

agcaatgcgt taattccatc atttcccata tataacctcc attttgtaat actggcattg 14880

ttgtgaagtt ggatttcact acaatattat tattagggca aatatcacta cttgtaataa 14940

catgcacaaa tacaatgcca ttcaatttaa ttgtatgtat aactgcctta gccaaagcat 15000

tagttaactg tattaatttg tcagtatagc atgttatttt taacaatgct acttcatcat 15060

tgtcaaacaa attttgcaat ctaactttta tcatactcaa tgagttgctg cccatctcta 15120

accaaaggag taaaatttaa gtggtactta tcaaattctt atttgcccca ttttttggt 15180

ttacgcaagt ttgttgtacg cattttttcg cgt 15213

<210> 2
<211> 47
<212> DNA
<213> Artificial
<400> 2
aattggtacc taatacgact cactataggg acgagaaaaa aagtgtc 47

<210> 3
<211> 27
<212> DNA
<213> Artificial
<400> 3
ttaaacgcgt catcaaacta ttaactc 27

<210> 4
<211> 29
<212> DNA
<213> Artificial
<400> 4
aattacgcgt taagcattag gattgagtg 29

<210> 5
<211> 33
<212> DNA
<213> Artificial
<400> 5
ttaaggatcc gcgcgctatt attgcaaaaa gcc 33

<210> 6
<211> 27
<212> DNA
<213> Artificial
<400> 6
aattgcgcgc tttttaatga ctactgg 2

<210> 7
<211> 38
<212> DNA
<213> Artificial
<400> 7
ttaaggatcc gtacgttggg gcaaatgcaa acatgtcc 38

<210> 8
<211> 28
<212> DNA

<213> Artificial
<400> 8
ttaacccggg gcaaataaaa catcatgg 28

<210> 9
<211> 46
<212> DNA
<213> Artificial
<400> 9
aattcgtacg tattgttagt cttaatatct tagttcattg ttatga 46

<210> 10
<211> 32
<212> DNA
<213> Artificial
<400> 10
aattcccggg atttttttta ttaactcaaa gc 32

<210> 11
<211> 43
<212> DNA
<213> Artificial
<400> 11
ttaaacctgg taagatgaaa gatggggcaa atacaaaaat ggc 43

<210> 12
<211> 38
<212> DNA
<213> Artificial
<400> 12
aattggatcc accaggtctc tccttaattt taaattac 38

<210> 13
<211> 65
<212> DNA
<213> Artificial
<400> 13
aattcttaag ggaccgcgag gaggtggaga tgccatgccg acccacgcga aaaaatgcgt 60
acaac 65

<210> 14
<211> 28
<212> DNA
<213> Artificial
<400> 14
gtccgacctg ggcatccgaa ggaggacg 28

<210> 15
<211> 26
<212> DNA
<213> Artificial
<400> 15
acgtcctcct tcggatgccc aggtcg 26

<210> 16
<211> 68
<212> DNA
<213> Artificial
<400> 16
tcgtccactc ggatggctaa gggaataacc ccttggggcc tctaaacggg tcttgagggg 60
tttttttgc 68

<210> 17
<211> 71
<212> DNA
<213> Artificial
<400> 17
ggccgcaaaa aacccctcaa gacccgttta gaggccccaa ggggttattc ccttagccat 60

ccgagtggac g 71

<210> 18
<211> 29
<212> DNA
<213> Artificial
<400> 18
ttaactcgag ttattcatta tgaaagttg 29

<210> 19
<211> 27
<212> DNA
<213> Artificial
<400> 19
aattggtacc gggacaaaat ggatccc 27

<210> 20
<211> 27
<212> DNA
<213> Artificial
<400> 20
ttaatctaga ttgtaactat attatag 27

<210> 21
<211> 31
<212> DNA
<213> Artificial
<400> 21
aattggatcc ggggcaaata aatcatcatg g 31

<210> 22
<211> 29
<212> DNA
<213> Artificial
<400> 22
aattggatcc ggggcaaata caagatggc 29

<210> 23
<211> 31
<212> DNA
<213> Artificial
<400> 23
ttaactcgag attaactcaa agctctacat c 31

<210> 24
<211> 31
<212> DNA
<213> Artificial
<400> 24
aattggatcc ggggcaaata tgtcacgaag g 31

<210> 25
<211> 34
<212> DNA
<213> Artificial
<400> 25
ttaatctaga tcaggtagta tcattatttt tggc 34

<210> 26
<211> 26
<212> DNA
<213> Artificial
<400> 26
ttaatctaga agtaactact ggcgtg 26

<210> 27
<211> 44
<212> DNA

<213> Artificial
<400> 27
aattggatcc ggggcaaata caaacatgtc caaaaacaag gacc 44

<210> 28
<211> 32
<212> DNA
<213> Artificial
<400> 28
aattccatgg ggtccaaaac caaggaccaa cg 32

<210> 29
<211> 31
<212> DNA
<213> Artificial
<400> 29
aaaagtatac ttaatgtgat ttgtgctata g 31

<210> 30
<211> 44
<212> DNA
<213> Artificial
<400> 30
ttttgtatac tggcagctat aatctcaact tcacttataa ttgc 44

<210> 31
<211> 27
<212> DNA
<213> Artificial
<400> 31
aatttctaga tttttaatga ctactgg 27

<210> 32
<211> 31
<212> DNA
<213> Artificial
<400> 32
ttaatctaga cgttacgcga acgcgaagtc c 31

<210> 33
<211> 41
<212> DNA
<213> Artificial
<400> 33
aattaagctt accatggaca cgattaacat cgctaagaac g 41